Pancreas-specific aquaporin 12 null mice showed increased susceptibility to caerulein-induced acute pancreatitis

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1Department of Nephrology and 2Department of Pathology, Tokyo Medical and Dental University, Tokyo; 3Supportive Center for Brain Research, National Institute for Physiological Sciences and 4Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, Okazaki; 5Division of Gastroenterology, Department of Medicine, Nagoya University Graduate School of Medicine, Nagoya; 6Department of Medical Physiology, Meiji Pharmaceutical University, Tokyo; and 7Department of Medicine, Kidney Center, Tokyo Women’s Medical University, Tokyo, Japan

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Ohta E, Itoh T, Nemoto T, Kumagai J, Ko SB, Ishibashi K, Ohno M, Uchida K, Ohta A, Sohara E, Uchida S, Sasaki S, Rai T. Pancreas-specific aquaporin 12 null mice showed increased susceptibility to caerulein-induced acute pancreatitis. Am J Physiol Cell Physiol 297: C1368–C1378, 2009. First published September 2, 2009; doi:10.1152/ajpcell.00117.2009.—Aquaporin 12 (AQP12) is the most recently identified member of the mammalian AQP family and is specifically expressed in pancreatic acinar cells. In vitro expression studies have revealed that AQP12 is localized at intracellular sites. To determine the physiological roles of AQP12 in the pancreas, we generated knockout mice for this gene (AQP12-KO). No obvious differences were observed under normal conditions between wild-type (WT) and AQP12-KO mice in terms of growth, blood chemistry, pancreatic fluid content, or histology. However, when we induced pancreatitis through the administration of a cholecystokinin-8 (CCK-8) analog, the AQP12-KO mice showed more severe pathological damage to this organ than WT mice. Furthermore, when we analyzed exocytosis in the pancreatic acini using a two-photon excitation imaging method, the results revealed larger exocytotic vesicles (vacuoles) in the acini of AQP12-KO mice at a high CCK-8 dose (100 nM). From these results, we conclude that AQP12 may function in the mechanisms that control the proper secretion of pancreatic fluid following rapid and intense stimulation.

pancreatic acinar cell; knockout mouse; pancreatic exocrine function; cholecystokinin; exocytosis

THE EXOCRINE PANCREAS has a large capacity to secrete digestive fluid with 1.5–2.0 liters of pancreatic juice containing digestive enzymes secreted into the duodenum each day in humans (14). The pancreas exocrine gland consists of two morphologically and functionally distinct groups of epithelial cells, the acinar cells and the duct cells. The acinar cells generate the primary Cl−-rich fluid, which facilitates the transport of digestive enzymes, whereas the downstream duct cells secrete HCO3−-rich fluid to regulate the pH of the pancreatic juice. Since both cell types generate near-isotonic fluids, the transepithelial osmotic gradients driving the water flow must be small. It is thought, therefore, that the apical and basolateral water permeabilities of both the acinar and duct cell membrane would need to be high and would therefore require the presence of water channels, i.e., aquaporins (AQPs). AQPs are small-membrane proteins with six transmembrane domains that form pores that are highly permeable to water. To date, at least 13 members of the AQP family (AQPO–AP12) have been identified in mammals. These proteins are expressed in various fluid-transporting epithelia and endothelia, each with a distinct tissue-specific expression pattern.

Extensive studies have now been performed in the search for AQPs in the pancreas and have already revealed the existence of several of these proteins in this organ including AQP1, AQP5, AQP8, and AQP12. Northern blot analysis of the whole rat pancreas has previously revealed high levels of AQP1 and AQP8 mRNA (8). AQP1 also has been detected by immunohistochemistry in the plasma membrane of the centroacinar cells and in the apical and basolateral membranes of intercalated and intralobular duct epithelia in the human pancreas (3). AQP1 was further demonstrated to be present in the membrane fraction of isolated zymogen granules (ZGs) from the rat pancreas (4). AQP5 has been detected by immunohistochemistry in the apical pole of intercalated duct cells but not in the acinar cells of the human pancreas (3). AQP8 was detected by immunohistochemistry in the apical pole of acinar cells in the mouse pancreas (8) and showed the same distribution in the human pancreas (3).

We have recently cloned AQP12 from both the mouse and human pancreas as a novel member of this protein family (9). According to the genome database, counterparts of the AQP12 gene can be found in Caenorhabditis elegans and in several vertebrates such as rat and chicken. Although the precise role of AQP12 in vivo is still unknown, this aquaporin is specifically expressed in pancreatic acinar cells in mouse, and our previous in vitro expression studies have shown that AQP12 is not targeted to the plasma membrane but shows an intracellular localization (9). These findings suggest that AQP12 may reside in intracellular vesicles in pancreatic acinar cells and thus may be involved in their specific exocrine functions. To determine the possible roles of AQP12 in the exocrine function of the pancreas, we generated AQP12 knockout (AQP12-KO) mice and analyzed the resulting phenotype.

MATERIALS AND METHODS

Generation of AQP12 knockout mice. To generate AQP12 knockout mice, we designed a targeting vector comprising a 6.5-kb genomic DNA fragment containing intron 1 (left arm) and a 1.1-kb fragment containing the region after exon 3 (right arm) of the AQP12 gene (Fig. 1A). Exons 2 and 3 of the gene were replaced with a 1.8-kb neomycin cassette to

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facilitate positive selection. This targeting vector was linearized and electroporated into 129/SvJ/129/Sv-CP embryonic stem cells. Drug-resistant clones were then screened using PCR and Southern blotting, in which genomic DNA were digested with BamHI, electrophoresed, transferred to a nylon membrane, and hybridized with a genomic fragment probe (Fig. 1B). Targeted embryonic stem cells were injected into C57Bl/6 blastocysts, and chimeric mice were bred with C57Bl/6 mice to produce F1 heterozygotes. Offspring were genotyped by genomic PCR with the use of primer pairs specific for the wild-type allele (5'-CTG CAG CTC CTA ACA GAT CT-3' and 5'-TTC CTC ACA CAT GGG TGA CA-3', 1.4-kb product) and the knockout allele (5'-TGG GCA CAA CAG ACA ATC GG-3' and 5'-CAG CTT CCA CAT GCA TGT GT-3', 1.8-kb product) (Fig. 1C).

All animal experiments were approved by the ethics committee of Tokyo Medical and Dental University.

Northern blot analysis. Total RNA was extracted using RNAlater (Ambion, Austin, TX) and TRIzol reagent (Invitrogen, Carlsbad, CA) from the pancreatic tissue of wild-type (WT) and knockout (AQP12-KO) mice. Poly(A)+RNA was purified from 600 µg of total RNA using the Poly(A)+ isolation kit (Nippon Gene, Tokyo, Japan). Aliquots of Poly(A)+ RNA were then resolved on a 1% formaldehyde-agarose denaturing gel (1 µg/lane) and transferred to a nylon membrane overnight. The membrane was subsequently hybridized with the 32P[dCTP]-labeled DNA probe at 42°C for 16 h. The AQP12 probe (510 bp) was obtained by digestion of the pTD1 vector containing full-length mouse AQP12 cDNA with Kpn1 and Apa1.

RT-PCR. Total RNA from mouse pancreas was reverse-transcribed using Omniscript reverse transcriptase (Qiagen, Valencia, CA). PCR was performed using the following primers: 5'-TCT TCC TCT ACA CAT GGG TGA CA-3', 1.4-kb product) and the knockout allele (5'-TGG GCA CAA CAG ACA ATC GG-3' and 5'-CAG CTT CCA CAT GCA TGT GT-3', 1.8-kb product) (Fig. 1C). All animal experiments were approved by the ethics committee of Tokyo Medical and Dental University.

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Diet studies. Mice were challenged using two different dietary protocols: a high-fat diet and caloric restriction. Experiments were carried out using AQP12-KO and WT mice of a matched background, sex, and age. For the standard diet group, standard mouse chow (containing 5% fat, NMF; Nippon Bio-Supp Center, Tokyo, Japan) was used. The high-fat diet group was fed with mouse chow containing 60% animal fat (NMF+60% lard; Nippon Bio-Supp Center). In
the standard diet group, mice were weaned at 5 wk after birth and given free access to water and standard mouse chow. In the high-fat diet group, mice were weaned at 4 wk after birth and also given free access to water and the high-fat diet. For the caloric restriction group, mice at 7 wk were given standard mouse chow but were restricted to 50 kcal per week. In all groups, body weights were recorded regularly, and blood samples were collected 3 wk after the initiation of the experimental diet.

Light and electron microscopy. For light microscopy, mouse pancreatic tissue samples were fixed by immersion in 4% paraformaldehyde for 12 h, embedded in paraffin, thin sectioned, and stained with hematoxylin and eosin. For electron microscopy, pieces of mouse pancreas no larger than 2 mm³ were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. After postfixation in 1% osmium tetroxide on ice and staining with 1% tannic acid and 1% uranyl acetate, tissue samples were dehydrated and embedded in Epon 812. Thin sections were then counterstained with lead citrate for analysis.

Analysis of amylase contents of mouse pancreas by immunoblotting. For immunoblotting, pancreatic protein extracts from AQP12-KO mice and their WT littermates (5 mice per group) were electrophoresed (SDS-PAGE, 10–20%) and transferred to a nitrocellulose membrane. The amylase contents were then determined by immunoblotting with a rabbit anti-human amylase primary antibody (1:1,000 dilution; Sigma-Aldrich, St. Louis, MO) with a rabbit anti-actin antibody (1:200 dilution; Cytoskeleton, Denver, CO) used as a control.

Isolation of the rough endoplasmic reticulum from the pancreas. For detailed analysis of the intracellular localization of AQP12, we isolated the rough endoplasmic reticulum (rER) from the rat pancreas and performed immunoblotting. To obtain the rER from the rat pancreas, we used an endoplasmic reticulum extraction kit (Imgenex, San Diego, CA) following the manufacturer’s instructions. Briefly, pancreatic tissue was homogenized in the supplied buffer and then centrifuged at 1,000 g for 10 min at 4°C to remove the nuclei. The supernatant was further centrifuged at 12,000 g for 15 min at 4°C to pellet out the mitochondria and broken cell debris. CaCl₂ solution was then added to the supernatant for precipitation, the supernatant was centrifuged at 8,000 g for 10 min at 4°C, and the pellet containing the rER-fraction was obtained. The rER fraction was submitted to immunoblotting with the anti-rat AQP12 antibody (1:1,000 dilution) using an anti-calnexin antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) as a control.

Water permeability of the mouse pancreas rER. To investigate the contribution of AQP12 to the water permeability of the pancreatic rER, we isolated this organelle from both AQP12-KO mice and WT littermates as described above. Osmotic water permeability was then measured by stopped-flow light scattering as described previously (23). Briefly, the rER preparation was suspended in 50 mM mannitol-Tris buffer (pH 7.4) and mixed for <1 ms with an equal volume of hyperosmolar buffer (500 mM mannitol-Tris buffer) to give a 225 mosmol/kgH₂O inwardly directed osmotic gradient. Ninety-degree light scattering was measured at a wavelength of 400 nm using a stopped-flow reaction analyzer (Applied Photophysics, Leatherhead, UK) and analyzed using SX.18MV Spectrometer software (Applied Photophysics). The time course of the vesicle volume is given by

\[ V(t) = V_0 + \int P_i(S/V_0) d\tau = V_0 + \int [C_1(t=0) - C_0] \]

where \( V(t) \) is the vesicle volume, \( V_0 \) is the molar volume of the water, \( P_i \) is the osmotic water permeability, and \( C_0 \) and \( C_1(t=0) \) are the initial vesicle surface-to-volume ratio, the initial intravesicular osmolality, and the constant solution osmolality. To calculate \( S/V_0 \), we measured the diameter of the ER vesicles using the electron microscopic negative stain method. Water permeability was computed using a vesicle \( S/V_0 \) of \( 3 \times 10^9 \) cm⁻¹.
AQP12-KO mice

Analysis of the AQP1 and AQP8, and pancreatic digestive enzyme expression levels in the pancreas of WT and AQP12-KO mice

Statistical analysis. Data are means ± SE. Statistical analysis was performed using the Student’s t-test. P values <0.05 were considered to be significant.

RESULTS

Generation of AQP12 knockout mice. A knockout targeting vector was constructed to replace exons 2 and 3 of the murine AQP12 gene with a neomycin cassette to enable subsequent positive selection. Three independent embryonic stem cell clones were obtained and injected into blastocysts derived from C57BL/6J mice, and correct gene targeting was confirmed by Southern hybridization (Fig. 1C). Intercross-breeding of heterozygotes was performed to produce WT, heterozygous, and KO mice (Fig. 1C). AQP12-KO mice were successfully produced as confirmed by Northern blot analysis (Fig. 1D). AQP12-KO mice were subsequently bred to produce WT, heterozygous, and KO mice (Fig. 1C).

Diet studies. The body weight of the mice at 5 wk of age was 19.1 ± 2.5 g (mean ± SE) for WT and 18.3 ± 2.6 g for AQP12-KO mice, but these differences were not significant. AQP12-KO mice also showed normal growth with no differences from WT mice when fed a standard diet (Fig. 2A). Even at 18 mo of age, the AQP12-KO mice showed no apparent differences in weight, reproduction, or survival rates compared with their WT counterparts. There were also no signs of any insufficiency in the pancreatic exocrine functions of the knock-out mice, such as weight loss or diarrhea.

To test the hypothesis that AQP12 plays a role in dietary fat processing, as observed for AQP1 in studies of the corresponding null mice (14), we weaned WT and AQP12-KO mice at 4 wk of age and placed them on a diet containing 60% fat. The mean body weights of the mice at weaning were 16.5 ± 2.6 g for WT and 16.5 ± 2.0 g for AQP12-KO, which was again not a significant difference. These mice subsequently showed a similar pattern of weight gain (Fig. 2B). For the caloric restriction study, we used 7-wk-old mice that had grown normally on a diet of standard chow. The initial mean weights were 22.3 ± 1.4 g for WT and 22.7 ± 1.8 g for AQP12-KO mice, which were not significantly different. The WT and AQP12-KO mice then showed similar levels of weight loss (Fig. 2C) following caloric restriction. As summarized in Table 2, the blood chemistries for both groups of mice showed no differences except for a significantly higher total cholesterol level in the AQP12-KO mice.

Microscopic analysis. Light microscopic analyses revealed no morphological abnormalities in the pancreatic tissue of the AQP12-KO mice (Figs. 3, A–D). Electron microscopy also detected no abnormalities in the size, number, or density of the ZGs in the AQP12-KO background (Fig. 3, E and F).

Amylase contents in the pancreas and analysis of pancreatic secretion. As determined by Western blotting, no difference in the amylase protein content of the pancreas was evident between WT and AQP12-KO mice (Fig. 4A). We also analyzed the expression levels of pancreatic digestive en-
zymes, including amylase, lipase, and CEL, using quantitative RT-PCR. CEL is a pancreatic enzyme known to be associated with cholesterol absorption and metabolism (13). However, although AQP12-KO mice have higher serum cholesterol levels than WT mice, we observed no differences in the expression levels of these three enzymes in the knockout animals (Table 1).

We then analyzed whether pancreatic secretion was affected by the loss of AQP12 using the duodenal loop method. Fluid samples that had been secreted into duodenal blind loops were

Table 2. Biochemical analysis of the blood in WT and AQP12-KO mice

<table>
<thead>
<tr>
<th></th>
<th>Standard (5% Fat) Diet</th>
<th></th>
<th>High-Fat (60%) Diet</th>
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<th>Caloric Restriction</th>
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<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
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<tr>
<td>Amylase, U/l</td>
<td>640±150</td>
<td>695±139</td>
<td>576±163</td>
<td>615±146</td>
<td>576±163</td>
</tr>
<tr>
<td>Lipase, U/l</td>
<td>35.6±8.2</td>
<td>41.2±9.0</td>
<td>41.1±16.0</td>
<td>35.7±6.0</td>
<td>41.1±16.0</td>
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<tr>
<td>Albumin, g/dl</td>
<td>3.00±0.43</td>
<td>3.24±0.26</td>
<td>3.13±0.32</td>
<td>3.02±0.32</td>
<td>3.13±0.32</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>182±44</td>
<td>187±44</td>
<td>174±32</td>
<td>158±30.6</td>
<td>174±32</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>80.0±10.1</td>
<td>102.8±6.1 †</td>
<td>151.3±28.4</td>
<td>172.6±27.1 †</td>
<td>151.3±28.4</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>60.8±24.0</td>
<td>50.8±20.3</td>
<td>26.6±14.7</td>
<td>21.6±12.6</td>
<td>26.6±14.7</td>
</tr>
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</table>

Data are means ± SE (standard diet: n = 14 WT and 18 KO mice; high-fat diet: n = 16 WT and 12 KO mice; caloric restriction: n = 6 WT and 7 KO mice). *P < 0.05; †P < 0.001 vs. WT under the same conditions.

Fig. 3. Light and electron microscopy analysis. Pancreatic tissues from WT mice (A, C, and E) and AQP12-KO mice (B, D, and F) were examined under a light microscope after staining with hematoxylin and eosin (A and B, original magnification, ×200; C and D, original magnification, ×640) or by electron microscopy (E and F, original magnification, ×3,000).
collected at 5 min after PBS injection, and the fluid volumes and amylase and lipase concentrations were then measured. Before CCK-8 stimulation, $155 \pm 11.2 \mu l$ (including PBS) of fluid were collected from the WT mice ($n = 5$), whereas $161 \pm 7.8 \mu l$ were collected from the AQP12-KO animals ($n = 6$). After CCK-8 administration, however, these volumes increased to $251 \pm 29.3 \mu l$ in WT and $271 \pm 13.6 \mu l$ in AQP12-KO mice, but the differences in these increased values are not significant (Fig. 4B). Both amylase and lipase secretion also were found to be unaffected by AQP12 deletion, either before or after CCK-8 administration (Fig. 4, C and D). We conclude from these data that no obvious changes in the fluid flow rate or in the secretion of amylase and lipase result from the loss of AQP12.

**Induction of acute pancreatitis.** Our analyses thus far had revealed no phenotypic differences between WT and AQP12-KO mice, and we therefore examined the pancreatic tissue of the AQP12-KO mice under pathological conditions. For this purpose, we treated the mice with caerulein, which is a well-established method for inducing self-limiting acute pancreatitis (1, 2). At 9 and 24 h after the injection of this agent, blood samples were collected and analyzed, and the animals were subsequently euthanized for histological examination. In the WT mice, the serum amylase and lipase levels were found to be elevated at 9 h but to have recovered to near normal levels at 24 h, consistent with previous reports (1, 6, 12, 29) (Fig. 5A). At 9 h in the AQP12-KO mice, however, both the serum amylase and lipase levels were significantly higher compared

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**Fig. 4.** Measurement of the amylase contents in the pancreas and analysis of pancreatic secretions. **A:** immunoblotting analysis of pancreatic homogenates to determine the amylase levels ($n = 5$ WT and 5 KO mice). **B:** measurements of the loop fluid volumes. **C:** amylase activity levels in the loop fluid. **D:** lipase activity levels in the loop fluid. Data in **B–D** are means ± SE ($n = 5$ WT and 6 KO mice). CCK, cholecystokinin.

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**Fig. 5.** Analysis of a mouse model of acute pancreatitis (caerulein induced). **A:** determination of the serum amylase and lipase levels of WT and AQP12-KO mice after induction of acute pancreatitis. Both the amylase and lipase levels at 9 h were significantly elevated in AQP12-KO mice compared with WT mice. Data are means ± SE ($n = 8$ WT and 9 KO mice at 9 h and 6 WT and 7 KO mice at 24 h). *P < 0.05; **P < 0.01 vs. WT. **B:** assessment of the histological damage of the pancreas at 24 h after the induction of acute pancreatitis. Sections were stained with hematoxylin and eosin. The pancreatic tissue in AQP12-KO mice (b and d) showed more severe pathological damage than that in WT mice (a and c), including edema, inflammation, and elevated necrosis of the acinar cells (a and b, original magnification, ×100; c and d, original magnification, ×640). **C:** histological grading of acute pancreatitis. The scores for both edema and acinar necrosis as well as the total score for pathological damage were statistically higher in AQP12-KO mice. Data are means ± SE ($n = 5$ WT and 6 KO mice). *P < 0.01; **P < 0.001 vs. WT.
with WT mice (Fig. 5A). As shown by histological examination, acute pancreatitis in WT mice could be characterized by mild edema and inflammation, but necrosis of the acinar cells was only barely evident (Fig. 5B, a and c). On the other hand, the onset of induced pancreatitis in the AQP12-KO mice was found to be associated with far more severe pathological damage, including obvious signs of edema, inflammation, and necrotic acinar cells (Fig. 5B, b and d). The data shown in Fig. 5C summarize the severity of this acute pancreatitis, as assessed by a pathologist in a blind manner. The scores for both edema and acinar necrosis, as well as the total score for the pathological damage, were statistically higher in the AQP12-KO mice. These findings indicate that AQP12-KO mice are more susceptible to caerulein-induced pancreatitis.

Two-photon excitation imaging of mouse pancreatic acini. Two-photon excitation imaging facilitates a greater depth penetration than confocal imaging in intact pancreatic acini (16), thus enabling visualization of the sequential processes of ZG exocytosis in exocrine glands. Since isolated acini were immersed in a solution containing fluid-phase fluorescent polar tracer, exocytosis could be observed. We thus performed these imaging analyses in the AQP12-KO mice. At a low concentration (100 pM) of CCK-8 stimulation, isolated acini of both WT and AQP12-KO mice showed a very similar pattern, i.e., normal exocytosis of individual ZGs (data not shown). However, at a high concentration (100 nM) of CCK-8 stimulation, the ZGs of AQP12-KO mouse acini were observed to merge with each other and form larger vacuoles within 10 min of the initiation of stimulation, whereas the acini of WT mice showed frequent exocytosis of ZGs of normal size (Fig. 6A). To confirm this phenomenon, we prepared hematoxylin and eosin sections in a manner similar to those used in the two-photon experiments. As shown in Fig. 6B, more vacuoles were observed in AQP12-KO mice than in WT mice (the number of vacuoles per 100 acinar cells was measured at 2.3 ± 2.9 and 21.6 ± 13.0 in WT and AQP12-KO, respectively; n = 4, P < 0.01).

Localization of AQP12 in the rat pancreas. We examined the cellular localization of AQP12 in the rat pancreas because we were unable to raise an antibody against mouse AQP12, although several attempts were made. We found, however, that one of the antibodies generated against the carboxyl terminus of AQP12 could detect rat AQP12. The specificity of the AQP12 antibody was confirmed by immunofluorescence and immunoblotting of rat pancreas extracts with the antigen absorption test (Fig. 7, A and B). As shown in Fig. 7C, AQP12 was found to localize at the basal side of the intracellular organelles of acinar cells close to the nucleus but not in either...
the duct cells or the islet cells. This finding is consistent with our previous in situ hybridization results for AQP12 in the mouse pancreas (9). In rats treated with CCK-8 (Fig. 7C, right), AQP12 distribution was more dispersed toward the apical side of the acinar cells compared with the untreated animals (Fig. 7C, left). Immunogold labeling of the rat pancreas further revealed that AQP12 is predominantly located in the rER in the absence of CCK-8 treatment (data not shown). Furthermore, in CCK-8 treated rats, AQP12 was mainly present in the rER and also on the membranes of ZGs near the rER (Fig. 8A). A negative control without incubation with the primary antibody is shown in Fig. 8B. These findings suggest that AQP12 may be involved in the mechanisms underlying the proper generation and maturation of ZGs. To confirm this intracellular localization of AQP12, we isolated the rER from the rat pancreas and performed immunoblotting, using calnexin as the ER marker. As shown in Fig. 9, robust expression of AQP12 was found in the rER.

Water permeability of the mouse pancreas rER. Finally, to investigate whether AQP12 contributes to the water permeability of the rER membranes in the mouse pancreas, we measured this permeability using vesicles obtained from the AQP12-KO and WT mouse pancreas. Osmotic water permeability ($P_f$) was measured over a time course of 90° scattered light intensity in response to a rapidly imposed osmotic gradient. There was no significant difference among the diameters of the vesicles in each preparation (99.8 ± 3.78 × 10⁻⁷ cm for WT and 100.2 ± 3.24 × 10⁻⁷ cm for AQP12-KO). The calculated $P_f$ for the rER membranes of WT and AQP12-KO mice was measured at 13.0 ± 1.5 × 10⁻³ and 15.1 ± 1.6 × 10⁻³ cm/s, respectively, which is not a significant difference.

**DISCUSSION**

AQP1, AQP8 and AQP12 have been shown to be expressed in pancreas acinar cells, and previous studies of AQP1 and AQP8 knockout mice have shown that these animals manifest only mild phenotypes. AQP1 null mice show a dietary fat processing defect, but there are multiple factors that could account for this finding given that AQP1 is expressed at many sites. Pancreatic secretion is not significantly affected by AQP1 deletion (14). The loss of AQP8 does not appear to affect the pancreatic function in mice either, and these AQP8 null mice have shown few phenotypic abnormalities despite the broad expression of this protein channel (26). We thus speculated that AQP12 might play an important functional role as a water channel in pancreatic acinar cells. Although we did not find any compensatory increase in the expression of other AQPs in AQP12 null mice, functional redundancy could well account for the apparent lack of any phenotypes in pancreatic AQP null mice.

In our present study, we generated and analyzed AQP12-deficient mice to evaluate the potential pancreatic exocrine functions of this aquaporin isoform. Although the loss of AQP12 did not affect the overall pancreatic exocrine function in mice under a normal breeding environment, AQP12-KO mice showed a more severe pathology resulting from CCK-8 analog-induced pancreatitis than WT mice. Furthermore, when exposed to a high concentration of CCK-8, the pancreatic acini...
of AQP12-KO mice formed more numerous and larger exocytotic vesicles (vacuoles) within a short period of time compared with WT mice. These findings indicate that the exocrine function of the pancreas is impaired in AQP12-KO mice when excessive stimuli are applied.

The formation of large vacuoles has been established as an important cellular hallmark of early pancreatitis (11, 19, 20, 24) and is the result of aberrant endocytosis of large postexocytic structures. Cytosolic vacuoles are possible sites for the intracellular and inappropriate activation of trypsinogen, which will lead to autodigestion and the development of pancreatitis (5, 7, 20). An important question to address in relation to this disease is the nature of the possible mechanisms by which the genetic deletion of a water channel might influence this formation of large cytosolic vacuoles. The primary function of pancreatic acinar cells is to synthesize, package, and secrete digestive enzymes (18). In these cells, digestive enzymes are stored in ZGs in the apical poles (18). When ZGs mature, they progressively reduce their volume and thus concentrate the digestive enzymes therein. For exocytosis to occur, it is thought that osmotic swelling of the granules must be initiated via a coupled influx of water and ions into the granule matrix (22). Thus the entire process for the formation, maturation, and exocytosis of ZGs is dependent on water transport across the membranes of the intracellular organelles. A decrease in the water flow into acinar cells may cause lower or negative intracellular pressure, and as a result, vacuoles may form more easily. Since the exocytosis of ZGs requires an outward water flow to release their contents into the lumen, a water flow defect may result in the accumulation and merging of ZGs within the acinar cells, thereby forming larger vacuoles. It has been reported that, in contrast to supramaximal CCK-8 or caerulein administration, supramaximal levels of CCK-58 do not cause pancreatitis. This is possibly attributable to the previously reported observation that supramaximal CCK-58 maintains pancreatic acinar water secretion, which is essential for exocytosis of activated zymogens (25). Clearly, therefore, a disruption to the water flow is one of the contributing factors in the onset of pancreatitis.

Another question to address in relation to AQP12 is the precise point at which this water channel exerts its effects during the functional processing of ZGs. We could not successfully generate an antibody against mouse AQP12 despite many attempts to do so. Fortunately, however, one of the mouse antibodies we did raise was immunoreactive against rat AQP12. Our immunofluorescence and immunoelectron microscopic analysis of the rat pancreas revealed that AQP12 is

![Fig. 8. Immunoelectron microscopic analysis of the rat pancreas following CCK-8 treatment. A: AQP12 was predominantly observed in the endoplasmic reticulum (ER) and also on the membranes of zymogen granules near the ER. B: negative control (incubation without the primary antibody).](image)

![Fig. 9. Immunoblotting analysis of the rat pancreas rough ER (rER) fraction. Calnexin was used as the ER marker. Protein, AQP12 protein synthesized in vitro; WL, whole lysate from the rat pancreas. Robust expression of AQP12 was found in the rER.](image)
localized at the basal side of the intracellular organelles of acinar cells, mainly at the ER. Immunoblotting of the isolated rER fraction from the rat pancreas also confirmed this finding. When we then measured the water permeability of rER membrane of the pancreas, no significant difference was found in the $P_f$ values between WT and AQP12 null mice. This result indicates the possibility that other AQPs are present on the rER membrane and that the contribution of AQP12 to water permeability of this membrane is small under basal conditions. This also is consistent with the lack of a phenotype in AQP12 null mice under a normal breeding environment.

However, upon CCK-8 stimulation, AQP12 distribution was found to be more dispersed toward the apical side of the cell, with some of these proteins also present on the ZG membranes close to the ER. This intracellular localization pattern of AQP12 suggests that it has a role in the early stages in ZG formation and maturation. When cells are stimulated by secretagogues and the rapid utilization of zymogen granules is thereby initiated, more AQP12 is recruited to the ZGs from the ER. Hence, under excessive stimuli, the absence of AQP12 likely impedes the intracellular water transport process, leading to the formation of functional aberrant ZGs, an event that cannot be reversed by compensatory activities of other AQPs.

Since it is difficult to prepare adequate samples of pancreatic ER or ZG fractions for $P_f$ measurement in the presence of pancreatitis, we were unable to demonstrate the abnormal water permeability of AQP12 null intracellular organelles under the influence of rapid and intense secretagogue stimulation.

Although most members of the AQP family are localized in the plasma membrane and their roles in transcellular water transport pathways have been well characterized, some AQPs are also localized at intracellular sites. AQP6 was revealed previously to reside in intracellular membrane vesicles and was suggested to play a role in the acidification of these bodies (27, 28). AQP1 has been detected in isolated ZG membranes of pancreatic acinar cells and shown to be involved in the regulation of secretory vesicle swelling (4). Moreover, AQP1 and AQP6 have been reported to be involved in synaptic vesicle swelling (10). Both of these previous studies indicate that secretory vesicle swelling is a requirement for the regulated expulsion of intravesicular contents during secretion and that intracellular AQPs may be part of the molecular components of this. However, none of these observations have been definitively shown to have any physiological significance in vivo.

AQP11 and AQP12 are the most recently identified AQPs and have been classified as members of a new AQP subfamily, the “subcellular AQPs.” AQP11 is expressed in intracellular sites, most likely at the ER, in a wide variety of tissues (15). AQP11 null mice develop intracellular vacuoles at multiple sites, such as the kidney proximal tubules, hepatocytes, and intestinal epithelial cells, and eventually die from polycystic kidney disease. This was the first report to show that the disruption of an intracellular AQP results in the onset of a pathological state in vivo. Our current findings concerning the increased susceptibility to pancreatitis resulting from a loss of AQP12 represent only the second demonstration to date that an intracellular AQP has a pathophysiological role in vivo. Our data thus shed new light on the pathophysiology of acute pancreatitis and also on the functions of intracellular AQPs in general.

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GRANTS

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REFERENCES


